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INTESTINAL COLONIZATION BY ENTEROTOXIGENIC ESCHERICHIA COLI.(U)
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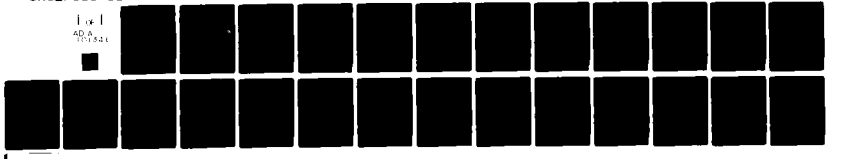

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INTESTINAL COLONIZATION BY ENTEROTOXIGENIC *Escherichia coli*

FINAL REPORT

HARLEY W. MOON

September, 1980

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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National Animal Disease Center
U.S. Department of Agriculture, and
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Intestinal colonization and adhesion by enterotoxigenic E. coli is mediated by specific types of pili. These pili are antigenic and can be used in diagnosing enterotoxigenic E. coli infections. They are also good protective antigens. When pregnant dams are vaccinated parenterally or orally with pili on live piliated bacteria, they secrete antibodies against the pili in their milk. Neonates suckling dams so vaccinated are passively protected against fatal challenge by enterotoxigenic E. coli. Pili are also good candidate protective antigens for the development of vaccines to protect by active immunization.

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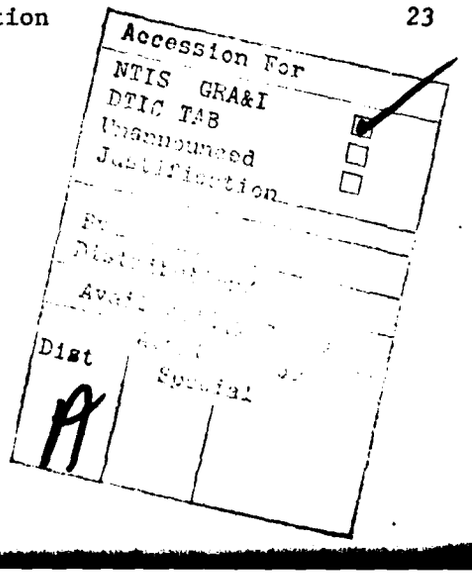

Summary

This study was designed to test the following hypotheses: 1) That enterotoxigenic E. coli (ETEC) characteristically colonize mammalian small intestine by adhering to the epithelial surface; 2) that adhesion is mediated by certain specific pili; 3) That pili can be used as protective antigens in vaccines for ETEC. The results documented in the publications listed in the Chronological Bibliography support all 3 of these hypotheses.

The experimental animal models used in the study were ETEC infections in neonatal pigs and calves. These models permitted studies on host adapted ETEC in intact natural hosts. The results will be summarized according to the 3 hypotheses listed above.

1. ETEC Colonize by Adhesion

Earlier work in this laboratory and in others had shown that certain ETEC which produce K88 type pili, colonize swine intestine by adhesion. Work documented in Bibliography publications 3, 5, 8, 14, and 18 demonstrates that this is a general characteristic of ETEC infections. Pig and calf ETEC which do not produce K88 all consistently colonized by adhesion and loss of adhesive ability correlated with loss of colonizing ability and virulence.

2. Adhesion is Mediated by Specific Pili

Work done in other laboratories had demonstrated that K88 pili mediate the adhesion of some ETEC to swine intestine. Work documented in Bibliography publications 4, 6, 7, 12, 14, and 18 demonstrates that pili of types 987P and K99 mediate adhesion of ETEC which produce them. $987P^+$ ETEC adhere to swine intestine and $K99^+$ ETEC adhere to swine, cattle, and sheep intestine. Pili convey some of the species specificity which is characteristic of ETEC. Species specificity is not absolute in that $K99^+$ ETEC colonize swine, cattle, sheep, and mice (Bibliography publication 17). Publication 12 also documents the specificity of the receptor - pilus interactions with intestinal epithelium and demonstrates that intestinal epithelium from a single individual has specific receptors for at least 3 different types of pili. Publications 6 and 18 demonstrate that some types of pili are produced more readily in vivo than in vitro. Publication 18 demonstrates that most (> 90%) of the ETEC which currently affect neonatal swine in the US produce 1 of 3 antigen types of pili, but that there are ETEC which are virulent for swine but which don't produce any of these 3 pili.

3. Pili are Protective Antigens

Work documented in Bibliography publications 9, 11, 12, 13, 15, 16, and 19 demonstrate that purified pili can be used as protective antigens in parenteral vaccines for ETEC infections in suckling neonates, or as living orally administered vaccines in the same system. This approach provides a way around the limitation to anti-enterotoxin immunity presented by the existence of non-antigenic heat-stable types of enterotoxin. Preliminary efforts to protect by oral vaccination with pili in a killed vaccine were not successful (publication 19).

4. Contributions Not Directly Related to the Major Objectives of the Study

Bibliography publications 1 and 2 demonstrate that mitomycin C enhances the production of heat-labile enterotoxin in vitro.

Publication 3 demonstrates that the capsules of some, but not all, ETEC are necessary for intestinal colinization and virulence.

Publications 10 and 17 demonstrate that intestinal transit of infant mice accelerates with age and ambient temperature and that these variables effect enterotoxin assays and studies on bacterial colonization in infant mice. A simplification of the infant mouse assay for ST based on these observations is presented (publication 10).

5. Manuscript Not Yet Published

Publication 19 has been submitted for publication. A copy of that manuscript is included in the body of this report.

Foreword

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences - National Research Council.

Protection Against Enteric Colibacillosis in Pigs Suckling Orally Vaccinated Dams: Evidence for Pili as Protective Antigens

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This work was conducted with the technical assistance of S.M. Skartvedt and R.A. Schneider. The work was supported by SEA, USDA, and by U.S. Army Medical Research and Development Command Grant 17-17-C-5014.

SUMMARY

Pregnant gilts were vaccinated orally with Escherichia coli that produce pilus antigens K99 or 987P. The vaccines were live or dead enterotoxigenic E. coli (ETEC) or a live rough non-ETEC strain which has little ability to colonize pig intestine. Pigs born to the gilts were challenged orally with $K99^+$ or $987P^+$ ETEC, which did not produce heat-labile enterotoxin or flagellae and which produced somatic and capsular antigens different from those of the vaccine strains.

Control gilts had low titers of serum and colostral antibodies against pilus antigens, and their suckling pigs developed a high incidence of fatal diarrhea after challenge. Serum antibody titers against pilus antigens of the vaccine strains increased after vaccination with live ETEC, and the colostral antibody titers of these gilts were higher than those of controls. Pigs suckling such gilts were more resistant than controls to challenge by ETEC of the homologous pilus type. This resistance was not attained when vaccine and challenge strains were of different pilus types, and it could not be attributed to enterotoxin neutralization by colostrum. In contrast to the live ETEC vaccines, the live rough non-ETEC and dead ETEC vaccines stimulated little or no production of antibody against pili, and pigs in these vaccine groups remained highly susceptible to challenge.

The results support the hypothesis that pili can be protective antigens in oral ETEC vaccines. It was suggested that in the system reported, protection depended on living bacteria for the production of pilus antigens in vivo or for the transport of pilus antigens across intestinal epithelium.

Pili or fimbriae are minute, proteinacious, filamentous appendages that are produced by a variety of bacteria. Certain types of pili produced by enterotoxigenic Escherichia coli (ETEC) mediate adhesion to epithelium and facilitate colonization of the small intestine of mammalian hosts by ETEC.[1] Three antigen types of pili, designated K88, K99, and 987P, have been implicated in colonization of the small intestine of newborn pigs by ETEC. K99 pili also facilitate intestinal colonization in calves and lambs. Suckling pigs,[2-4] calves,[5] and lambs[6] whose dams had been vaccinated parenterally with purified cell-free preparations of pili were protected against fatal diarrheal disease caused by ETEC bearing pili homologous to those in the vaccines. In the above experiments[2-6] although ETEC were recovered from the immune sucklings, protection correlated with impaired colonization (reduced numbers and adhesion) of the small intestine by the ETEC.

Pregnant swine that were fed live ETEC for 3 days subsequently protected their suckling pigs against challenge with the strains of ETEC fed to the dams.[7] Protection correlated with impaired colonization of the small intestine by the ETEC. These observations have been the basis for use of live oral ETEC vaccines in the field.[8] The applied program depends on the use of strains of ETEC that are autogenous to the herd in question, and the protective antigens involved are not known.

In addition to local passive lactogenic immunity as described above,[2-7] live ETEC can induce active intestinal immunity. Human volunteers challenged orally with ETEC were immune to subsequent challenge by the same strain but susceptible to a challenge by a heterologous strain of ETEC.[9] The protective antigens involved are not known. They appear to act independently of bactericidal antibodies and antibodies agains enterotoxin or somatic antigen.

Some ETEC produce pili in the intestine more readily than they do in vitro.[3,10] Furthermore, people with diarrhea caused by ETEC produced antibodies against the pili of the infecting strains.[11] The objective of the work reported here was to test the hypothesis that pilus antigens can be protective by way of oral vaccination with ETEC. The tests of the hypothesis involved vaccination of pregnant swine. Their sucklings were subsequently challenged by ETEC that were heterologous to, but of the same pilus type as, the vaccine strains.

Materials and Methods

Animals - Forty-nine crossbred swine (gilts) from 2 herds were moved to the National Animal Disease Center 6 weeks before their anticipated farrowing dates. They were confined in individual isolation rooms for the last 2-4 weeks of gestation. These gilts and 356 pigs subsequently born to them were the subjects of this study.

E. coli - The strains used and some of their characteristics are listed (Table 1). Strains that produced K99 or 987P pili and heat-stable (ST) but not heat-labile (LT) enterotoxin were selected to avoid antitoxic immunity[12] and hereditary resistance to colonization by $K88^+$ ETEC.[13] Cultures were grown aerobically in broth[a] (16 hrs at 37°C).

Vaccination - Two weeks before their anticipated farrowing dates, gilts were fasted for 24 hrs and then fed cultures or broth mixed with 1.4 kg of feed, on each of 3 successive days. Gilts were vaccinated (principals) by feeding each of them 200 ml of fresh broth culture/day (10^{11} E. coli/day).[7]

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[a]Trypticase Soy Broth-BBL, Becton, Dickinson & Co., Cockeysville, MD.

Bacteria in two vaccines were killed (dead vaccines) by adding formalin (to 0.5% by volume) and incubating at 37°C for 4 hrs. Killed cells were added to bring one of the dead vaccines up to 10^{13} E. coli/gilt/day. These additional cells were obtained by centrifugation of cultures produced as usual. Control gilts (non-vaccinated) were fed 200 ml of sterile broth/day.

Serology - Blood samples were taken from gilts 4 days before they were vaccinated and again 10 days after the last day of vaccination. Colostrum samples were taken from the gilts on the day they farrowed. Blood serum and colostral whey were stored frozen and subsequently tested for antibodies against K99 or 987P. Some samples were tested for antibodies against LT and ST enterotoxins. All samples to be tested were encoded by persons other than those who conducted the tests. After the tests had been completed, samples were decoded to identify gilt number, vaccination record, sample type and date.

Titers of K99 antibody were determined by an enzyme linked immunosorbent assay (ELISA).[14] The K99 antigen for the assay was prepared[15] from E. coli strain Troyer (Table 1). Standard K99 antiserum prepared against E. coli strain K12($K99^+$) and absorbed as previously reported[16] was conjugated to alkaline phosphatase and used as the indicator antiserum. Antibody titers were recorded as the reciprocal of the highest dilution of serum or whey that blocked detectable binding of the indicator antiserum to the antigen (blocking assay).[14]

Titers of 987P antibody were determined by a bacterial agglutination technique (tube system) as described for titrating antibody against

K antigens of E. coli.[17] E. coli strain 987 in the piliated phase was used to prepare P^+ antigen; and the encapsulated but non-piliated mutant (I36) of strain 987 was used to prepare P^- antigen.[18] Neither of these antigens was agglutinated by antiserum containing 09 antibodies. The positive control serum was the monospecific 987P antiserum prepared and absorbed as previously reported.[3] This serum agglutinated the P^+ but not the P^- antigen. None of the samples from gilts agglutinated the P^- antigen at dilutions of 1:10 or higher. The reciprocal of the highest dilution of serum or whey which caused detectable agglutination of the P^+ antigen was recorded as the titer of 987P antibody. The adrenal cell culture assay was used to test for neutralizing antibodies against LT enterotoxin.[19] The infant mouse assay and ST produced by E. coli strain 431 were used to test for neutralization of ST enterotoxin.[20] Crude ST was mixed (1:1) with samples of whey or saline and incubated at 37°C for 1 hr and then tested in infant mice (at the effective dose 50 for un-neutralized ST).

Challenge Inoculation - Newborn pigs (0-7 hrs old) were identified individually by ear notches, weighed, and then challenged before they suckled. Each pig was inoculated with 10^9 viable E. coli of strain 987 or 10^{10} viable E. coli of strain 431 (Table 1). These strains do not produce flagellae and the experiments were designed (Tables 2 and 3) so that they were of different somatic and capsular antigen types than the vaccine strains. These inocula were prepared in advance as previously reported[2] and stored at -70°C in 10% glycerol until used. The inoculum for each pig was suspended in 20 ml of broth and given by gavage. All pigs in a litter were given the same strain and were allowed to suckle at will

immediately after inoculation. Pigs that were small (<800 gms) or weak or that suckled before inoculation were removed when first observed and were deleted from the experiments.

Clinical Observations - Gilts were examined for diarrhea 1 day after each vaccine feeding. Suckling pigs were examined once a day, starting 16-24 hrs after inoculation, for 5 days after inoculation. The incidence of diarrhea, deaths due to diarrhea (diarrhea, dehydration, loss $\geq$ 10% of initial body weight) and the weight gain of survivors were recorded.

Results

Clinical Response of Gilts - All gilts ate the feed-vaccine mixtures given to them. None of the gilts developed diarrhea at any time during the experiments.

Serology - There were low titers of K99 and 987P antibodies in serum and colostrum of control gilts and in serum taken from principal gilts before vaccination (Tables 2 and 3).

Serum K99 antibody titers of gilts vaccinated with live Troyer strain increased after vaccination (Table 2). Titers of K99 antibody in colostrum from the gilts were higher than those in serum from the same individuals and markedly higher than those in colostrum from controls. Titers of K99 antibody in serum and colostrum from gilts vaccinated with dead Troyer strain were similar to those from controls, even when the dose of bacteria was increased 100-fold. The K99 antibody titers in serum and colostrum from gilts vaccinated with live K12 strain tended to be greater than titers in controls but less than titers in gilts vaccinated with live Troyer strain.

Vaccination with live 74-5208 strain induced antibodies against 987P in serum and colostrum (Table 3). Titers of 987P antibody were higher in serum 2 and colostrum of gilts vaccinated with strain 74-5208 than in controls or gilts vaccinated with strain 431. Titers of 987P antibody in colostrum were greater than those in serum from the same individuals.

Colostrum samples from 27 gilts were tested for neutralizing antibody against ST and LT. None of the 27 samples of colostral whey contained ST neutralizing antibody. The reciprocal geometric mean LT neutralizing titers of these 27 samples (vaccine group:titer/no. of gilts tested) were as follows: Control:70/9, 74-5208:12/10, live Troyer:10/4 and K12:18/4.

Response to Challenge with Strain 431 - The incidence of fatal diarrhea was high in pigs in the control group (Table 2). Thos- that survived gained little or no weight, and some still had diarrhea 5 days after inoculation.

Pigs in the live Troyer vaccine group had lower incidences of diarrhea and death, had diarrhea of shorter duration and more weight gain among survivors than did controls (Table 2). Incidences of fatal diarrhea were high in the dead Troyer and K12 vaccine groups (Table 2). The incidence of death was somewhat lower in the 74-5208 group than in controls, but the incidence and duration of diarrhea and weight gain of survivors in this group were similar to that in controls.

Response to Challenge with Strain 987 - The incidence of fatal diarrhea was high in pigs in the control group. The incidences of diarrhea and death were lower, the duration of diarrhea was shorter, and weight gains were higher in pigs in the 74-5208 group than in controls (Table 3). Pigs in the 431 vaccine group responded nearly as severely as the controls did.

Discussion

Gilts vaccinated orally with live ETEC produced colostral antibodies against pilus antigens of the vaccine strains. Pigs suckling such gilts were more resistant than controls to challenge with ETEC that were heterologous to, but of the same pilus type as, the vaccine strains. These results support the hypothesis that pilus antigens can be protective antigens in oral vaccines against ETEC infections. It is unlikely that vaccine induced protection (live Troyer, Table 2 and live 74-5208, Table 3) was due to antitoxic immunity. The strains used for vaccine and challenge produce ST but not LT, and ST is considered to be non-antigenic and not neutralizable by LT antitoxin.[12] Colostrum from gilts in the protected group did not contain ST neutralizing activity, and LT antitoxin titers of these samples were not higher than those of controls. Furthermore, this protection was not duplicated by vaccinating with ETEC that were completely heterologous to the challenge strains (live 74-5208, Table 2 and live 431, Table 3). The somewhat lower incidences of death in these 2 groups than in controls is probably more an indication of the variability of the system than of vaccine induced, non-specific immunity.

Presumably, the immune response and protection reported here depended upon pilus antigens produced *in vivo*. Dead Troyer vaccine was ineffective even at the 10^{13} dose. The live K12 strain vaccine was also ineffective. However, this rough laboratory strain is poorly suited for survival *in vivo*[21] and colonizes pig small intestine poorly in spite of carrying K99 antigen.[1] Peyer's patches have a mechanism for the enhanced uptake and transport of macromolecules from the intestinal lumen and are one site where orally administered antigens contact and activate the immune system.[22-24] There is evidence that stimulation of the secretory immune system by some bacteria depends on their ability to leave the intestinal lumen and to multiply and persist in Peyer's patches.[25] Such a dependency could explain why the dead Troyer and K12 vaccines were ineffective. It is conceivable that a few of the bacteria from the live Troyer and 74-5208 vaccines entered, multiplied, and persisted in Peyer's patches even though ETEC have little invasive ability and are mostly confined to the intestinal lumen.

In contrast to the results with pili reported here, some orally administered dead bacterial antigens can stimulate the secretory immune system in some circumstances,[26-28] and a dead oral vaccine can protect against ETEC infections in swine.[29] From the standpoint of vaccine development, effective dead preparations would be useful to have. Comparative studies on the location of pilus antigens and bacteria after oral vaccination with live and dead ETEC would contribute to the understanding of immunity to enteric infections.

TABLE 1 - Characteristics of *E. coli* Strains Used

Strain	Antigens				Source	Enterotoxin	References
	Somatic	Capsular	Pilus	Flagellar			
431	0101	K30	K99	NM*	Pig	ST+	16
Troyer	09	K35	K99	NM	Pig	ST	7,16
K12	Rough	-	K99	?	Laboratory‡	None	30
987	09	K103	987P	NM	Pig	ST	2
74-5208	020	K101	987P	NM	Pig	ST	2

*Non-motile.

+Produces heat stable enterotoxin detectable by infant mouse assay, but does not produce heat labile enterotoxin.

‡K99 plasmid introduced by manipulation in the laboratory.

TABLE 2 - K99 Antibody Titers of Serum and Colostrum from Gilts Vaccinated Orally with E. coli and Response of Their Suckling Pigs to Challenge with E. coli Strain 431 (Which Produces Pilus Antigen K99)

Vaccine*					K99 antibody+			Response of pigs (%)			
			No. of		Serum			Diarrhea			
Strain	State	Pilus	Gilts	Pigs	1	2	Colostrum	Day 1	Day 5	Death day 1-5	Weight change‡
Control	Sterile broth	None	6	46	1	1	8	98	18	50	4
Troyer	Live-10^{11}	K99	6	47	1	233	3073	64	0	9	34
Troyer	Dead-10^{11}	K99	5	44	1	3	21	98	21	39	18
Troyer	Dead-10^{13}	K99	5	41	1	2	6	98	44	56	-3
K12	Live-10^{11}	K99	4	22	1	18	117	100	0	68	29
74-5208	Live-10^{11}	987P	5	26	1	2	2	96	21	23	2

*Vaccines were 10^{11} or 10^{13} live or dead E. coli/day for 3 days.

+Geometric means of reciprocals, serum 1 was collected 4 days before vaccination, serum 2 was collected 10 days after vaccination and colostrum was collected on the day of farrowing and challenge 6-19 (mean 13.2) days after vaccination.

‡Mean % change from initial body weight by those that survived to day 5.

TABLE 3 - 987P Antibody Titers of Serum and Colostrum from Gilts Vaccinated Orally with E. coli and Response of Their Suckling Pigs to Challenge with E. coli Strain 987 (Which Produces Pilus Antigen 987P)

Vaccine*					987P antibody+			Response of pigs (%)			
			No. of		Serum			Diarrhea			
Strain	State	Pilus	Gilts	Pigs	1	2	Colostrum	Day 1	Day 5	Death day 1-5	Weight change
Control	Sterile broth	None	7	52	1	1	10	83	57	73	13
74-5208	Live-10^{11}	987P	7	53	3	182	1479	43	0	6	49
431	Live-10^{11}	K99	4	25	2	7	48	92	59	36	18

*Vaccines were 10^{11} live E. coli/day for 3 days.

+Geometric means of reciprocals, serum 1 was collected 4 days before vaccination, serum 2 was collected 10 days after vaccination and colostrum was collected on the day of farrowing and challenge 6-19 (mean 13.2) days after vaccination.

‡Mean % change from initial body weight by those that survived to day 5.

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